IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Giammaria SITAR

Serial No.: 09/937,137

Filed: September 21, 2001

Examiner: Vera Afremova

Art Unit: 1651

Atty. Dkt.: 3026-101

For: A METHOD FOR THE SEPARATION OF FETAL CELLS FROM THE MATERNAL PERIPHERAL BLOOD

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

I, Giammaria Sitar, am the inventor of the above referenced application.

The Office rejected all pending claims under 35 U.S.C. §102(a) as being anticipated by Sitar et al., Cytrometry, April 1, 1999, Vol. 35, No. 4, pages 337-45.

I believe that I am the inventor of the cell separation method disclosed in this paper under the headings "Blood Sample Collection and Nucleated Cell Separation" and "Separation of mononuclear cells by isopyknic gradient centrifugation" on pages 338 to 339 of this paper.

The other listed authors of this paper, Siliva Garagna, Maurizio Zuccotti, Cristina Falcinelli, Laura Montanari, Alessandro Alfei, Giovanbattista Ippoliti, Carlo Alberto Redi, Remigio Moratti, Edoardi Ascari and Antonino Forabosco did not work with me on the described cell separation method. These co-authors

were either physicians who simply gave me maternal blood or cell biologist and geneticians who investigated if indeed fetal cells were present in the cell fraction I isolated from maternal blood using my cell separation method via, for example, PCR.

I, Giammaria Sitar, declare that all statements made herein that are based on my own knowledge are true and all statements made on information and belief are believed to be true. I acknowledge that willful false statements are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Giammaria Sitar

Giammaria Sitar

Date: 05/09/2005

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I, Giammaria Sitar, am a professor of medicine at the University of Pavia, Italy. I have been working on the subject of separating different cell types from blood since 1974 and have co-authored more than ten papers that deal with this subject.

The presently claimed invention requires that the claimed tissue culture mixture containing peripheral maternal blood has a pH of 6.4 to 6.6. This pH range will allow for the separation of fetal nucleated red blood cells (NRBCs) from maternal blood cells such as monocytes and lymphocytes which have cells densities very similar to NRBCs (Haematologica 1997,82: 5-10: "Characterization of the biophysical properties of human erythroblasts as a preliminary step to the isolation of fetal erythroblasts from maternal peripheral blood for non-invasive prenatal genetic investigation"). The claimed pH range ensures that the NRBCs become lighter and maternal blood cells with similar density distribution profiles become heavier so that they can be readily separated during subsequent

discontinuous density gradient centrifugation. This fact allows that NRBCs can be separated in a single centrifugation step. On information and belief, in all other procedures that I am aware of, the initial step of discontinuous density gradient centrifugation of maternal blood does not provide an enrichment of NRBCs but is performed to remove the bulk of red blood cells from maternal blood (there are about 125 BILLIONS red blood cells in 25 ml maternal blood) to avoid an overload of the system in the procedure that is subsequently used.

The below supports that the maternal blood cells containing mixtures that are subjected to centrifugation disclosed in U.S. Patents 5,641,628 and Patent 5,676,849 will not attain a pH within the presently claimed pH range. This supports that the methods disclosed in these patents will not allow for the separation of NRBCs from maternal blood cells or their isolation according to the presently claimed invention.

U.S. Patent No. 5,641,628 to Bianchi et al. discloses in example 10, starting in column 22, a method for detecting fetal stem cells in maternal circulation. On information and belief, the initial step of discontinuous density gradient centrifugation (column 22 line 44-45) is limited to removing red blood cells, while the true isolation technology is described to be the flow sorting of fluorescent cells (column, 22 line 50). In particular, the patent describes that mononuclear cells, isolated by discontinuous density gradient centrifugation, were incubated with monoclonal antibodies directed antigens expressed on precursors progenitor cells and flow sorted by FACS (Fluorescent activated cell sorter). Based on information I obtained during my work in the field of cells separation, I believe it to be technically impossible to use FACS if red blood cells are not previously removed.

Example 10 discloses collecting venous blood (20ml) in citrate dextrose (ACD-A) and subsequent centrifugation. I believe that Bianchi adds tissue culture medium such as standard liquid form of RPMI 1640 before separation

since this is, according to information I obtained during my work in the field of cell separation, standard procedure. It is my belief that it is clear that Bianchi uses citrate dextrose as anticoagulant. On information and belief, the normal percentile of most anticoagulants, in particular ACD, generally added to blood is not more than around 15%. While ACD has a pH of 4.4, a mixture of 3.75 ml of ACD (15%) with 21.25ml peripheral blood, which has a pH of around 7.39, results according to information obtained during recent studies performed in my laboratory, in a pH of 6.8 – 6.9. In fact, according to information obtained during those recent studies in my laboratory, adding ACD in the range of about 12% to 18% to peripheral blood will result in a pH of 6.8 – 6.9.

In column 13, line 42, Bianchi discloses the use of RPMI 1640 medium containing lithium heparin (10IU/ml). The standard liquid form of RPMI 1640, which is, on information and belief, generally used as tissue culture medium, contains bicarbonate and/or HEPES as buffers. On information and belief, only specialized formulations such as the 10x concentrated solution, the powered version or the modified version will not contain either sodium bicarbonate and/or HEPES as a buffer (see attached printout from Sigma-Adrich website). These buffers will prevent any substantial reduction in the pH of blood subsequent to, for example, the addition of ACD as an anticoagulant. Thus, on information and belief, if standard tissue culture medium and ACD is added to peripheral maternal blood, the pH will lie even higher than 6.8 -6.9, probably in the range of pH 7.0 - 7.4.

I believe that column 13 of Bianchi confirms that mononuclear cell separation is indeed only an initial step since she writes about the discussed prior art (line 54-55) "An advantage to this particular technique is that an initial step which results in mononuclear cell isolation is not added".

Bianchi discloses the isolation of a mononuclear cells layer after centrifugation. On information and belief, such a mononuclear cell layer will not

only contain fetal NRBCs, but also many maternal mononuclear cells such as monocytes and lymphocytes. This further supports that Bianchi's method does not allow for the isolation of NRBCs/their separation from maternal blood cells as presently claimed. Indeed discontinuous density gradient centrifugation is only a preliminary step. On information and belief, Bianchi's true separation is not based on density gradient centrifugation but on FACS (Fluorescent activated cell sorter).

U.S. Patent 5,676,849 to Sammons et al. discloses a method for the enrichment of fetal cell population from maternal whole blood samples. The Example spanning columns 10 and 11 includes two centrifugation steps. The first one is described in column 10, lines 36 to 62. Here 40ml blood is collected in an anticoagulant similar to those described earlier in column 6, lines 55 to 60, namely CPD (2.55g d-glucose, 2.63g sodium citrate, 0.327g citric acid and 0.222 monobasic sodium phosphate in 100 ml distilled water). The so collected blood is layered on HISTOPAQUE-1119 and centrifuged. Sammons explicitly states that CPD is used as an anticoagulant. Again the goal of this first centrifugation step is to remove red blood cells from a maternal blood sample. While I have not personally worked with CPD, I believe from my work with ACD, that the solution prepared in this first step of the example in column 10, lines 36 to 62, does not have the pH of the tissue culture mixture currently claimed. I believe that it is almost certain that such a mixture will not necessarily have a pH in the claimed range. My belief is based, among others, on my observation that blood cells in the claimed tissue culture mixture having pH of 6.4 to 6.6 generally begin to die after 24hrs. In column 6, lines 51 to 65, Sammons discloses that the blood collected in one of the anticoagulants listed, including CPDA, can be stored for 4 day at 4°C, which, based information obtained during my studies, implies that if this type of collected blood is used within 24hrs as Sammons suggests in this paragraph, this collected blood has a pH higher than 6.6, most likely higher than 7.0. Also, blood, even if not combined with a standard tissue culture medium, has a natural buffering capacity, which, on information and belief, would prevent

a drop of the pH of blood for some time even if acids are added, unless a specific effort is made such by the addition of non-buffered culture medium.

In the second centrifugation step described in column 10, line 63 to column 11, line 1, PBS (phosphate buffered solution) is added to the nuclear fraction obtained from the above described centrifugation step and the mix is then centrifuged. PBS has generally a pH of 7.2 to 7.4. The mix of the nuclear fraction and PBS thus has a pH well above the claimed range. On information and belief, in Sammons, the NRBCs only get truly separated during the charge flow separation (CFS) step disclosed in column 11, lines 2 to 12.

I, Giammaria Sitar, declare that all statements made herein that are based on my own knowledge are true and all statements made on information and belief are believed to be true. I acknowledge that willful false statements are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Giammaria Sitar

May 19, 2005

8360 Prof. GIAMMARIA SITAR
STR GMR 47R14 H501E
Medicina Interna III
IRCCS POLICLINICO S.MATTEO PV

Life Science
+ Antibody Explorer
+ Automation
Books
+ Cancer Research
− Cell Culture
− Product Lines
Antibody Expression
Classic Media & Salts
ECACC Cell Lines
Embryo Culture
Equipment
Gene Therapy
Primary Human Culture
Protein Expression/CHO
Reagents & Supplements
Sera
Serum Free Media
Stem Cells
SyntheChol NS0
TrypZean Solution
Vaccine Production
+ Product Highlights
+ Key Resources
+ Cell Signaling
Custom Synthesis
Drug Discovery
+ Molecular Biology
+ Plant Biotechnology
+ Proteomics and Protein Expr.
+ Life Science Quarterly

CLASSIC MEDIA AND SALTS

Cell Culture

Basal Salt Mixtures :: Dulbecco's Media :: Ham's Nutrient Mixtures :: MCDB
MEM Media :: Medium 199 :: MegaCell™ Media :: Other Classic Media :: RPI

RPMI Media

RPMI-1640 was developed by Moore et. al. at Roswell Park Memorial Institute, hence RPMI. The formulation is based on the RPMI-1630 series of media utilizing a bicarbon system and alterations in the amounts of amino acids and vitamins. RPMI-1640 mediu used for the culture of human normal and neoplastic leukocytes. RPMI-1640, when pr supplemented, has demonstrated wide applicability for supporting growth of many type cells, including fresh human lymphocytes in the 72 hour phytohemaglutinin (PHA) stimu

Product #	Product Name	Description	Application
R 0883	RPMI-1640 Medium	• Liquid • With sodium bicarbonate • Without L-glutamine; supplement with 0.3 gm/L L-glutamine • Sterile-filtered • Endotoxin tested • Cell culture tested	Same as R 8758, excep glutamine must be adde investigator.
R 8758	RPMI-1640 Medium	• Liquid • With sodium bicarbonate and L-glutamine • Sterile-filtered • Endotoxin tested • Cell culture tested	Complete liquid version RPMI-1640 formulation Moore et. al. in 1967. Developed for growth of and neoplastic leukocyte blood lymphocytes). Ge supplemented with seru
R 1145	RPMI-1640 Medium (10x)	• Liquid • Without L-glutamine, folic acid and sodium bicarbonate • Supplement with 0.3 gm/L L-glutamine, 0.001gm/L folic acid and 2.0 gm/L sodium bicarbonate at 1x • pH 1.8-2.2 at 10x • Sterile-filtered • Endotoxin tested • Cell culture tested	This is the concentrated of R 8758. The L-glutamine and so bicarbonate are added t after dilution to 1x and p
R 6504	RPMI-1640 Medium	• Powder • With L-glutamine • Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate. • Formulated at 10.4 grams of powder per liter of medium. • Cell culture tested	Related to R 5382, RPM Hybri-Max.
R 7509	RPMI-1640 Medium Modified	• Liquid • With sodium bicarbonate	Phenol red has been sh

		• Without phenol red and L-glutamine; supplement with 0.3 gm/L L-glutamine. • Sterile-filtered • Endotoxin tested • Cell culture tested	with the growth of some densities. Use this medium when stem cells or when grow densities. Also recommended for i diagnostics use.
R 5886	RPMI-1640 Medium HEPES Modification	• Liquid • With 25 mM HEPES and sodium bicarbonate • Without; L-glutamine; supplement with 0.3 gm/L L-glutamine. • Sterile-filtered • Endotoxin tested • Cell culture tested	Same as R 8758, excep buffer has been added. HEPES increases the b the medium and raises t buffering range. Recommended for cells better at a pH above 7.2
R 4130	RPMI-1640 Medium HEPES Modification	• Powder • With L-glutamine and 25 mM HEPES • Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate. • Formulated at 16.4 grams of powder per liter of medium. • Cell culture tested	Same as R 8758, excep buffer has been added. HEPES increases the b the medium and raises t buffering range. Recommended for cells better at a pH above 7.2
R 7638	RPMI-1640 Medium Dutch Modification	• Liquid • With 1 gm/L sodium bicarbonate and 20 mM HEPES • Without L-glutamine; supplement with 0.3 gm/L L-glutamine. • Sterile-filtered • Endotoxin tested • Cell culture tested	Same as R 8758, excep concentration of sodium has been cut in half and has been added. HEPES increases the b the medium and raises t buffering range. Recommended for cells better at a pH above 7.2
R 7388	RPMI-1640 Medium Modified	• Liquid • With 20 mM HEPES and L-glutamine • Without sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate. • Sterile-filtered • Endotoxin tested • Cell culture tested	Same as R 8758, excep buffer has been added. HEPES increases the b the medium and raises t buffering range. Recommended for cells better at a pH above 7.2
R 8755	RPMI-1640 Medium Modified	• Powder • With L-glutamine • Without phenol red and sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate. • Formulated at 10.4 grams of powder per liter of medium. • Cell culture tested	Phenol red has been sh with the growth of some densities. Use this medium when stem cells or when grow densities. Also recommended for i diagnostics use.
R 7755	RPMI-1640 Medium Auto-Mod?	• Powder	Modified for autoclaving

		• Without L-glutamine and sodium bicarbonate; supplement with 0.3 gm/L of L-glutamine and 2.0 gm/L sodium bicarbonate. • Formulated at 10.3 grams of powder per liter of medium. • Cell culture tested	
R 1383	RPMI-1640 Medium	• Powder • With L-glutamine • Without glucose and sodium bicarbonate; supplement with 2.0 gm/L sodium bicarbonate. • Formulated at 8.4 grams of powder per liter of medium. • Cell culture tested	Without glucose, allows add own energy source.
R 7513	RPMI-1640 Medium Modified	• Liquid • Without sodium bicarbonate • Without L-methionine, L-cystine, and L-glutamine • Supplement with 0.652 gm/L L-cystine-2HCL, 0.015 gm/L L-methionine, and 0.3 gm/L of L-glutamine • Sterile-filtered • Endotoxin tested • Cell culture tested	Formulated without sulfu amino acids. Recommended for meta using radio-labeled sulfu amino acids.

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The use of non-physiological conditions to isolate fetal cells from maternal blood

Giammaria Sitar[a,*], Bruno Brambati[b,c], Marina Baldi[d], Laura Montanari[c], Massimo Vincitorio[a], Lucia Tului[c], Antonino Forabosco[f], Edoardo Ascari[a]

[a]*Department of Medicine Policlinico San Matteo and University of Pavia, Italy*
[b]*First Department of Obstetrics and Gynecology, University of Milan, Italy*
[c]*Center of Prenatal Diagnosis, Italy*
[d]*Consultorio di Genetica, Rome, Italy*
[e]*Department of Obstetrics and Gynecology, University of Pavia, Italy*
[f]*Division of Genetics, University of Modena, Italy*

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Abstract

Fetal cells are always present in maternal blood starting in the first trimester of pregnancy, however a rapid, simple, and consistent procedure for their isolation for prenatal non-invasive genetic investigation is still lacking. Sensitivity and recovery of fetal cells is jeopardized by the minute amount of circulating fetal cells and their loss during the enrichment procedure. We report here a single-step approach to isolate fetal cells from maternal blood which relies on the use of non-physiological conditions to modify cell densities before their separation in a density gradient and in a newly developed cell separation device. Isolated fetal cells have been investigated using cytochemistry, Soret band absorption microscopy, monoclonal antibodies for ε- and γ-chain-Hb, monoclonal antibody for i-antigen, and by fluorescence in situ hybridization (FISH). Fetal cells were always detected in all 105 maternal blood samples investigated and fetal aneuploidies were correctly diagnosed by FISH, in a pilot study of pathological pregnancies, in fetal cells isolated from maternal blood obtained either before or after invasive procedure.

Keywords: Ion channels; Erythroblast; Stem cell; Fetal cells

Introduction

A long sought goal of medical genetics has been the replacement of currently used invasive procedure of embryo-fetal cell sampling by isolating fetal cells from maternal blood.

Hematopoietic cells arise first in the third week of human ontogeny inside yolk sac-developing blood vessels, then, 1 week later and independently, from the wall of the embryonic aorta and vitelline artery [1]. It is during the fourth week of gestation that erythroblasts enter into the embryo, soon thereafter hematopoietic cells begin to colonize, the newly forming liver, which serves as the major site of hemopoiesis throughout the remainder of the first trimester. During this stage of pregnancy, fetal hemopoiesis is mainly constituted by nucleated red blood cells (NRBCs) and $CD34^+$ stem cells [2].

Evidence exists for a two-way cell traffic across the fetal–maternal interface [3] and Krabchi et al. [4] have offered conclusive data that fetal cells are always present in maternal blood, their number being between 2 and 6 fetal cells/ml maternal blood. This very low number of fetal cells represents the most formidable obstacle that must be

* Corresponding author. Department of Medicine, University of Pavia, Policlinico San Matteo, Clinica Medica 3 Viale Golgi, Pavia 27100, Italy. Fax: +39 382 526223.
E-mail address: g.sitar@smatteo.pv.it (G. Sitar).

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overcome to obtain a sufficient number of fetal cells for reliable genetic investigation.

Almost any procedure available in experimental cell research for cell enrichment has been tried to isolate these cells out of maternal blood: density gradient centrifugation [5], fluorescent activated cell sorting (FACS) [6,7], magnetic activated cell sorting (MACS) [8], charge flow separation [9], micromanipulation [10,11], avidin–biotin columns [12]. All these multi-steps procedures require many manipulations in which some/many fetal cells are lost.

Comparative analysis of these different procedures has been the object of several reviews [13]. Despite all these efforts, a simple and efficient procedure is not yet available in clinical practice for routine testing.

In a previous investigation, we have shown that fetal NRBCs, from cord blood, can be isolated up to purity exploiting their different behavior, compared to all other nucleated cells, in non-physiological conditions [14]. We have simplified and improved this procedure to make it suitable to the isolation of fetal cells from maternal blood, which is technically far more demanding due to their minute amount in maternal blood.

Since NRBCs are only a subset of fetal cells entering maternal circulation and in view of the fact that stem cells are substantially represented in fetal blood [15,16], we also investigated the presence of CD34+-positive cell in the isolated cell fractions.

We have additionally analyzed the developmental expression of ε- and γ-chains of hemoglobin, and of i-antigen in the isolated cells from maternal blood during the progression of pregnancy. FISH has been used to evaluate male fetal cells and aneuploid cells in a pilot study of pathological pregnancies.

Materials and methods

Samples

Blood samples were obtained from women attending the Prenatal Diagnosis Unit of the private practice of the two obstetricians contributing this investigation. All women were previously informed and their consent was obtained.

The present investigation was performed after institutional review board approval.

The gestational age at sampling was calculated from the first day of the last menstruation period and confirmed by fetal ultrasonography.

Maternal blood was obtained either before chorionic villous sampling (CVS) or before amniocentesis into Na-EDTA anticoagulant. A total number of 105 blood samples were investigated from women with normal pregnancy before any invasive procedure, 56 of them were obtained from women in the 1st trimester of gestation, 49 from women in the 2nd trimester. While completing this series of samples, we also investigated eight cases of pathological pregnancies as diagnosed by invasive procedures, obtaining blood right before pregnancy termination, and eight cases with ultrasound abnormalities (nuchal thickening and/or other abnormalities) before any invasive procedure.

Cord blood was obtained at delivery to serve as a positive control, while blood from female non-pregnant laboratory personnel served as negative controls.

Red blood cell volume investigation in non-physiological medium

Twenty-five-milliliter control peripheral blood was added with an equal volume of 1 × medium 199- with Earle salts (pH 5.6, 268 mOsm/l) obtained diluting a 10× solution with water, either in presence or absence of ACD-A (Na citrate, citric acid and dextrose). The osmolarity of these solutions was adjusted using NaCl 20 mEq/10 ml.

Fetal NRBCs isolation from cord blood

Twenty-five milliliters of cord blood or maternal blood was transferred into a 60 ml tissue culture flask containing an equal volume of 1× medium 199- with Earle salts obtained diluting a 10× solution. Immediately after, 7.5 ml of ACD-A was added. This diluted blood has a pH of 6.2–6.4, NaCl was eventually added to raise the osmolarity up to 310–320 mOsm/l. Flasks were left overnight at 4°C. The following day, diluted blood was layered over different Biocoll (Biochrom, Berlin, Germany) solutions having increasing density of 1.070, 1.077, 1.080, and 1.082 g/l to investigate isolation efficiency and yield.

Fetal cell isolation from maternal blood by density gradient centrifugation

Twenty-five milliliters of maternal blood was transferred into a 60-ml tissue culture flask containing an equal volume of 1× medium 199-with Earle salts obtained diluting a 10× solution. Immediately after, 7.5 ml of ACD-A was added. This diluted blood has a pH of 6.2–6.4, NaCl was eventually added to raise the osmolarity up to 310–320 mOsm/l. Flasks were left overnight at 4°C. The following day, diluted blood was overlaid onto a Biocoll solution having a density of 1.082 g/l into a cell separation device (Fig. 1) previously described [17] and modified by the introduction of two lateral channels terminating into the cavity of the device, to make easier collection of cells floating at the interface between blood and the separating medium. Blood occupies the upper part of the internal chamber down to the level of the lateral outlet ports, below which is the separating medium. Centrifugation was run for 20 min at 400 × *g*. To avoid distribution disturbances, the rotor was slowly accelerated manually according to the time span suggested by Leif et al. [18] and the run was stopped without braking. The cell fraction floating at interface between plasma and Biocoll was collected by introducing at the base of the

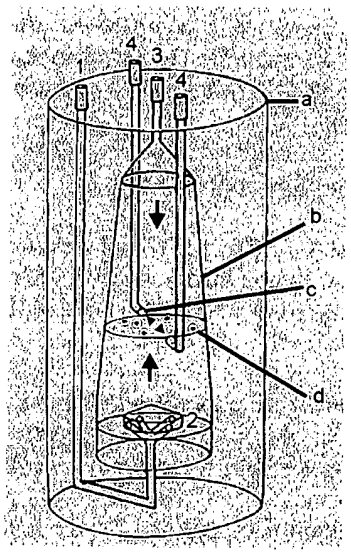

Fig. 1. A schematic view of the separation device (a) used to isolate fetal cells from maternal blood. The internal (90 ml volume) cavity (b) is first filled with separating medium through channel 1, and flow-deflector 2, up to channel 3, after which blood is introduced onto the separating medium through channel 3, by reversing the flow of the peristaltic pump, down to the level of the outlet ports (c) of channels 4. After centrifugation, cells floating at the interface (d) are retrieved by introducing simultaneously air through channel 3 and fluorinert FC-43 through channel 1(arrows), thus forcing out cells into lateral channels 4.

device an heavy fluid immiscible with water (Fluorinert FC-43, 3 M Company, St. Paul, USA) while simultaneously pumping air from the top, at the same rate, using the same two-channels peristaltic pump. Thus, the cell fraction floating at the level of lateral outports is compressed and forced out through these into the lateral channels (Fig. 1), without introducing any hydrodynamic disturbances at the interface.

A volume of 15 ml was obtained which contains only cells floating at the interface. Cells were centrifuged, washed once and enumerated using a Coulter Counter, before being left overnight at 37°C and 5% CO_2, to deplete the cellular fraction of platelets and monocytes which adhere to the flask plastic.

Isolated cells were investigated by PCR, cytochemistry, Soret band microscopy, immunocytochemistry, and FISH.

PCR

In 38 initial samples, cell suspensions obtained after separation were tested by nested PCR for fetal sex, with nested primers specific for amplifying the amelogenin gene present in both X and Y chromosomes. Primers pair AMG1-AMG2 described by Nakahori et al. [19] were used.

Slides preparation

Slides for benzidine staining, Soret band microscopy [20] and immunocytochemical investigations were prepared using a modified in-house-built 1 g sedimentation device previously described [21].

Slides for FISH investigation were obtained by cytocentrifugation using in-house-built cytobuckets with different deposition area appropriate to home either 0.5×10^6 or $0.5–2.0 \times 10^6$ cells, without cell overlapping.

Erythroblasts detection

For each sample, an aliquot of the cellular fraction was cytocentrifuged and stained by benzidine to confirm the presence of erythroblasts by morphology and cytochemistry. Slides, after fixation 10 min in methanol 100%, were placed in 0.05% benzidine reagent (6 ml of 5 mg benzidine in 3 ml ethanol, 1 ml of 30% hydrogen peroxide, 8 ml of 70% ethanol) 15 min in humid chamber, rinsed in tap water. Nuclei were counterstained with Giemsa solution. Microscopic examination showed cells with brown cytoplasm (hemoglobin) and a blue-stained nucleus which enabled differentiation between nucleated red blood cells and leukocyte background. Hemoglobin containing nucleated cells were further identified by light microscopy equipped with an interference filter for filtration of light in the Soret band, at 414 nm (Omega Co., Brattleboro, VT) allowing the detection of early NRBCs with a very low hemoglobin concentration, which are negative when stained with benzidine.

Immunostaining with ε- and γ-chain Mab

Immunostaining reaction for ε-chain and γ-chain hemoglobins was done in all peripheral blood investigated, after NRBCs isolation. Blood sample from umbilical veins of a newborn was obtained at birth as positive control and adult NRBC cells isolated from bone marrow were used as negative controls. Slides were fixed in methanol and acetic acid and incubated for 30 min at 37°C in 3% BSA in PBS as blocking reagent, quickly washed in PBS and incubated for 30 min at 37°C with either γ-chain-Hb MoAb directly conjugated with PE or ε-chain-Hb MoAb directly conjugated with FITC, both of Cortex Biochem (San Leandro, CA). The slides were than washed three times in 4× SSC at 37°C, counterstained with DAPI (0.1 μl/ml 4′,6-diaminidino-2-phenylindole) for 5 min at room temperature (RT) and viewed in the antifade solution DABCO. Slides were examined manually using DAPI, FITC and Rhodamine filters on a Zeiss Axioplane microscope. Nucleated cells that showed specific staining above the DAPI background stain were counted as positive.

Immunostaining with anti-i MoAb

Anti-i monoclonal antibody used in the present investigation has been previously described in details [22]. Isolated cells were resuspended in a final volume of 500 μl at a concentration of 1×10^6 cells/ml in RPMI 1640 containing 1% BSA and anti-i monoclonal antibody for 2 h. After two washings in RPMI 1640, cells were sedimented on glass slides at 1 g and left drying overnight at RT.

Table 1
Effect of non-physiological medium (osmolarity, pH and different ions concentration) on red blood cells mean corpuscolar volume

Sample	Osm	pH	Na	K	MCV
peripheral blood	292	7.39	142	4.1	92
25 ml blood + 3.75 ml ACD	311	6.81	154	3.0	96.9
25 ml blood + 25 ml medium	284	7.08	125	4.8	94
10 ml blood + 40 ml medium + 200 μl NaCl	276	6.78	127	5.0	100
25 ml blood + 25 ml medium + 1000 μl NaCl	370	7.1	183	5.2	96.4
25 ml blood + 25 ml medium + 7.5 ml ACD	285	6.3	137	3.9	106.8
25 ml blood + 25 ml medium + 2.5 ml ACD + 800 μl NaCl	355	6.8	179	4.7	99.5
25 ml blood + 25 ml medium + 5 ml ACD + 600 μl NaCl	346	6.4	173	4.6	105
25 ml blood + 25 ml medium + 7.5 ml ACD + 400 μl NaCl	321	6.4	162	4.4	105
25 ml blood + 25 ml medium + 10 ml ACD + 200 μl NaCl	313	6.4	157	4.2	109.4

ACD-A is citric acid, Na-citrate and dextrose.
Medium is 1 × 199-medium with Earle salts obtained diluting a 10× solution free of HCO_3.
NaCl is a 20 mEq/10 ml solution of NaCl.

Positive cells were detected using DAKO Envision System, Alkaline Phosphatase (DAKO, Copenhagen, DK) according to the manufacturer's instruction. Slides were counterstained with DAPI and visualized under a fluorescence microscope using appropriate filters.

Fluorescence in situ hybridization

X and Y chromosomal analysis was performed using directly labeled X- and Y-specific probes CEP X Spectrum Green/CEP Y-alpha Spectrum Orange probe mixture, (Vysis Downers Grove, IL, USA). Hybridization was performed according to the procedure described by Yan et al. [23]. To detect fetal cells with trisomy 21 and trisomy 18, slides were processed with TriGen LSI 21 SpectrumOrange and CEP 18 (α satellite) SpectrumOrange hybridizing centromere of human chromosome 18 (18p11.1–q11.1 Vysis). To detect fetal cells with trisomy 13, LSI 13 SpectrumGreen was used. Only intact cells that were not overlapping were chosen for the analysis. The hybridization efficiency of FISH was tested on NRBCs isolated from cord blood using this same procedure. A Leica DMRB fluorescent microscope equipped with Vysis filters and Q-FISH software has been used in FISH evaluation.

Results

Red blood cell mean corpuscolar volume (MCV) modification in different non-physiological conditions

After the addition of an equal volume of acid tissue culture medium to peripheral blood, a slight increase in MCV of red blood cells is observed, which is further increased by adding NaCl. The major increase of MCV is obtained when ACD-A is added to the system in presence of NaCl (Table 1).

NRBC isolation from cord blood

Table 2 shows NRBCs purity and yield when cord blood is separated by discontinuous density centrifugation over Biocoll solutions of increasing densities. NRBCs have been identified by benzidine staining and Soret band microscopy.

NRBCs and stem cells quantification, in the isolated cell fractions, on the basis of cytochemistry, immunocytochemistry, and Soret band microscopy

A mean number of 3.2×10^6 nucleated cells were obtained after isolation from maternal blood (range $1.2–6.8 \times 10^6$) which were aliquoted on five glass slides at a concentration of $2.5–5.0 \times 10^5$ cells/cm^2 using either a cytobucket with a small or a large deposition area depending on the number of cells available. All slides were screened manually. All samples investigated were positive for benzidine-stained nucleated cells, for ε- and γ- stained NRBCs and for i-antigen.

CD34+ cells were equally present in all isolated cell samples. In the blood of non-pregnant controls, NRBCs and CD34+ cells were always present, in no case, we detected cells positive for ε-Hb-NRBC, while in five cases, we detected cells positive for γ-Hb-NRBC.

Table 3 shows the number of NRBCs and stem cells detected in the isolated cell fraction and their immunophenotype. There was a good correlation between NRBCs counting either by benzidine staining or Soret band microscopy. This latter procedure has the major advantage of providing direct visualization of hemoglobin containing cells which can be later used for whichever procedure is required. Fig. 2 shows the presence of three NRBCs in the isolated cell fraction, stained by benzidine. Fig. 3 shows two ε-chain-Hb-positive nucleated cells stained using a FITC conjugated specific MoAb while Fig. 4 shows the presence of two CD34-positive cells in the isolated cell fractions. In few cases CD34+ cells were as many as 1% of the entire isolated cell fraction. Fig. 5 shows i-positive cells detected by a monoclonal specific antibody amplified with DAKO

Table 2

Separating medium (g/ml)	Erythroblasts purity(%)	Erythroblasts yield(%)
1.070	82 ± 6.4	15 ± 8
1.077	44 ± 4.8	45 ± 12
1.080	28 ± 3.8	65 ± 9
1.082	14 ± 6.2	84 ± 4

Isolation of erythroblasts from cord blood transferred into non-physiological conditions and separated by discontinuous density centrifugation on different densities.

Table 3
Number of NRBCs, ε+ and γ+ NRBCs, i-antigen + nucleated cells and CD34 + cells in the cell fraction isolated out of 25 ml peripheral blood and cord blood, on 1.082 g/ml

Weeks of gestation	Benzidine	Soret	ε-chain	γ-chain	i-antigen	CD 34-Ag
8–11 (n = 56)	14–334 (120 ± 95)	10–368 (148 ± 11)	5–48 (28 ± 18)	2–12 (6 ± 4)	>200	$>1 \times 10^3$
14–18 (n = 49)	20–452 (250 ± 152)	12–520 (280 ± 20)	2–22 (15 ± 6)	5–60 (35 ± 20)	>200	$>1 \times 10^3$
Cord blood at term (6)	12% of nucleated cells	15% of nucleated cells	0	>90% of NRBCs	60% of NRBCs	$>10^3$
Non-pregnant women (12)	1–20 (11 ± 7)	0–32 (15 ± 10)	0	2–6 (3 ± 1)	0–50 (22 ± 20)	$>1 \times 10^3$

Values are given in range (mean ± SD).

Envision system. Preliminary experimental data by FACS analysis show that a small percentage of isolated CD34+ cells co-express i-antigen (data not shown).

The frequency of ε-Hb versus γ-Hb-positive NRBCs showed an expected larger number of ε-Hb-positive cells in the first trimester, obtained before CVS, while the unexpected result was the presence of still some ε-Hb-positive cells in samples obtained in the second trimester of pregnancy before amniocentesis.

Presence by nested-PCR

In 37 out of the 38 samples analyzed by PCR, it was possible to correctly predict the sex of the fetus (Table 4). One sample gave a false-positive result (male in presence of female fetus). The presence of Y-positive cells in this latter case might be explained by the presence of a fetal cell derived from a former pregnancy.

Presence and number by FISH analysis

Table 5 shows the results of FISH analysis performed on 105 cell samples isolated from maternal blood using the described procedure. Analysis was carried out without knowing in advance fetal sex. The mean percentage of cells which showed one X and one Y signal or two X signals was >98% using this protocol [23]. This hybridization efficiency was comparable to the controls.

In samples out of the 1st trimester, 23 out of 24 male pregnancies were correctly predicted and in 30 out of 32 female pregnancies no Y-signals were found. In samples out of the 2nd trimester, 19 out of 21 male pregnancies were correctly predicted and in 26 out of 28 female pregnancies no Y-signals were observed. If the Y-signals observed in the female pregnancies were due to residual cells of previous male pregnancies or to specific signs of FISH procedure remains undetermined [24].

Out of 8 aneuploid pregnancies, identified with invasive procedures, trisomic cells (Fig. 6) were identified in all cases (Table 6) as well as in eight cases when blood was obtained before any invasive procedure.

Discussion

These studies were designed to develop a simple and efficient procedure for isolating fetal cells from maternal blood for prenatal non-invasive genetic investigation. By far, the simplest way to enrich for a cell type from a heterogeneous cell population is cell separation by density gradient centrifugation [25].

Simple and/or double density gradients have been described, to isolate fetal cells from maternal blood using either Percoll or Ficoll-solutions of different densities, reviewed by Prieto et al. [26] and Samura et al. [27]. Different results were obtained although a general agree-

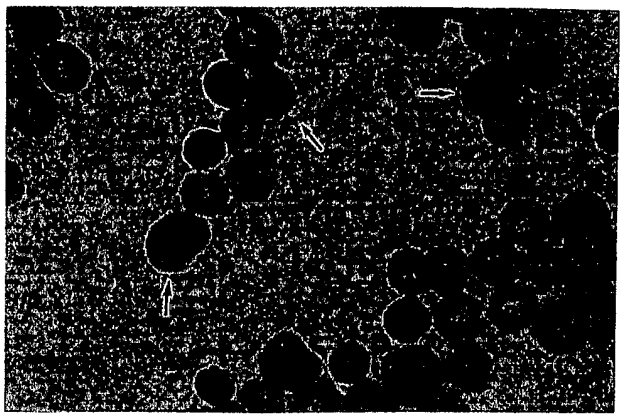

Fig. 2. Slide stained with DAB/Giemsa showing three nucleated cells with hemoglobin-containing cytoplasm (arrows) in the isolated cells from maternal blood.

Fig. 3. Slide stained with monoclonal antibody for ε-chain-Hb conjugated with FITC, nuclei are counterstained with DAPI. Two positive cells are present (arrows).

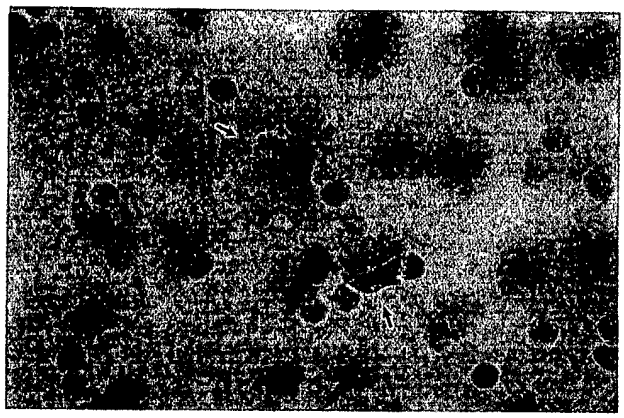

Fig. 4. Cells were stained with monoclonal antibody for CD34 then applied to microscope slide, fixed and stained with streptavidin-alkaline phosphatase and fast red (arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ment was reached that NRBCs are distributed both in the light and in the heavy portion of the gradient and that no difference exists in the density distribution profile of fetal and adult NRBCs. Since most nucleated cells present in human blood are lighter than 1.090 [28], the introduction of a very high density cut like 1.109 or 1.119 g/ml yields indeed most fetal cells present in maternal blood but without any enrichment, since all other nucleated cells are equally retrieved.

The removal of red blood cells by density gradient centrifugation on a Ficoll containing solution, in a standard centrifuge tube, produces inevitably a major loss of nucleated cells, usually between 30% and 50%, which remain entrapped between red blood cells which aggregate when they come in contact with Ficoll-containing solution. Since fetal cells, after density gradient centrifugation, are still dispersed between a large amount of maternal cells, further enrichment is required, usually by an antibody-dependent procedure like FACS or MACS. During these procedures, additional target cell loss takes place, thus, the eventual number of fetal cells available is often insufficient for genetic investigation [29].

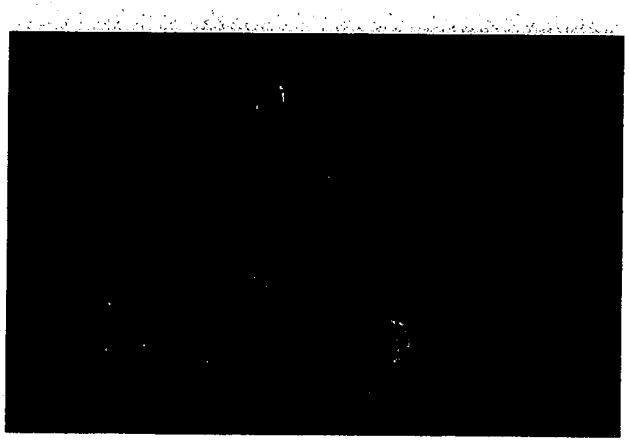

Fig. 5. Slide stained with monoclonal antibody for i-antigen. DAKO EnVision system-AP was used for immunocytochemical visualization.

Table 4
PCR analysis in 38 samples of maternal blood from normal pregnancies

Gestational age	No. of cases	PCR	Karyotype[a]
8–11 weeks	10	XX	XX
	1	XY	XX
	7	XY	XY
14–18 weeks	9	XX	XX
	11	XY	XY

[a] Chorionic Villus Sampling and amniocentesis are based on formal karyotype.

A single-step procedure would substantially reduces cell loss.

The strategy for isolating NRBCs from maternal blood, by a single density gradient centrifugation, must overcome two obstacles, the density distribution profile of NRBCs overlaps with that of maternal nucleated cells [30] and their extremely low number within a large bulk of maternal cells. The density of a cell is a function of the intracellular water content, which is determined by the chemical composition of the extracellular media.

Cells behave as perfect osmometer, when cell solute content (i.e., intracellular osmolality) or extracellular osmolarity is altered, rapid transmembrane water flow occurs and this equilibrium is restored. Because the plasma membrane is highly compliant water flow causes cell swelling or shrinkage [31,32]. It now appears that cells possess a wide array of volume-detector and volume-effector mechanism that respond selectively to both the magnitude and the nature of the volume perturbation, but these studies have disclosed a surprisingly diversity between different cell types in the nature of the ion transport system involved [33,34].

In this investigation, the redistribution of cell densities favorable to the isolation of NRBCs out of maternal nucleated cells has been obtained transferring maternal blood into a non-physiological medium of low pH and slightly high osmolarity. Under non-physiological conditions, cell volume perturbations are caused either by

Table 5
Fluorescence in situ hybridization analysis in 105 samples of maternal blood from normal pregnancies

Results				
Gestational age	No. of cases	FISH	Karyotype[a]	XY + cells[b]
8–11 weeks	30	XX	XX	–
	2	XY	XX	3–6 (4 ± 1)
	23	XY	XY	15–165 (90 ± 65)
	1	XX	XY	–
14–18 weeks	26	XX	XX	–
	2	XY	XX	1–8 (4 ± 3)
	19	XY	XY	12–98 (45 ± 30)
	2	XX	XY	–

[a] Chorionic Villus Sampling and amniocentesis are based on formal karyotype.

[b] XY + cells are those detected by FISH in the isolated cell fraction from maternal blood.

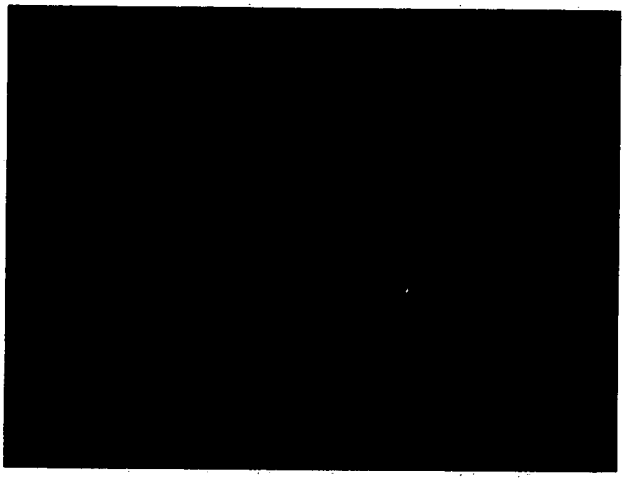

Fig. 6. Cells isolated from maternal blood before any invasive procedure. Digitalized microscopic image of stained nuclei (Dapi) showing three FISH signals for chromosome 21 in two nuclei, by fluorescent microscopy.

osmolarity and/or pH changes and the composition of extracellular fluids [35].

Strict regulation of the intracellular pH is essential for normal functioning of cells. Most cells are equipped with several mechanisms to protect the internal pH from modification of extracellular pH, but these mechanisms vary from cell line to cell line [36]. The peculiar behavior of erythroid cells in front of non-physiological extracellular medium prompted us to take advantage of this observation in the isolation of NRBC from maternal blood [14].

To optimize the isolation of NRBCs from maternal blood we investigated the modification of MCV of RBCs in different conditions of pH and osmolarity assuming that NRBCs behave similarly to RBCs. These preliminary experiments (Table 1) showed that lowering the pH of maternal blood, by diluting it with an acid medium, produces a substantial increase in MCV of RBCs. When ACD is further added, an additional increase of MCV is obtained even in the presence of high osmolarity. We have therefore selected those non-physiological conditions which provide the highest increase in MCV of RBCs (water intake) and therefore decrease their density. The selected density cut (1.082) was chosen based on previous investigations [30] on the density distribution profile of NRBCs.

Following these non-physiological conditions, a substantial increase of mononuclear cell densities is obtained and their yield, after separation on 1.082 g/ml, is reduced to less than 5% of their initial number.

Volume and density of cells transferred into non-physiological conditions are the final result of the interactions of many variables and the interaction between them is complicated [35]. Two major differences distinguish erythroid cells from all other cells present in human blood, the presence of anion exchange protein which is abundantly expressed on the cell membrane and the presence of hemoglobin. These two biological features of erythroid cells might explain why NRBCs become lighter in the non-physiological conditions used, while most other nucleated cells become heavier. The non-ideal osmotic behavior of hemoglobin [37], the change in the net charge on the impermeant cell ions with pH [38], the kinetics and turnover rate of the main monovalent ion transporters [39], and the operation of the Jacobs–Stuart cycle [40] are all now well characterized, although we are still unable to predict different behavior of erythroid cells in non-physiological conditions, which depend critically on the interactions of multiple functions [41].

Once a method had been devised to isolate fetal cells by a single density gradient centrifugation, it was then necessary to optimize cell recovery. For this purpose, we modified a previously described [17] cell separation device, which overcomes the drawbacks inherent in a standard centrifuge tube. The reader is referred to a previous review paper on this matter for a more extensive theoretical discussion of bioengineering details [17]. As additional advantage, cells floating at the interface are obtained in a reproducible way, by applying two compressive forces driving cells into the lateral channels and outside the cell separation device, eliminating the variability intrinsic in manual collection. The isolation procedure based on these two technical innovations provides a cell fraction were NRBCs and CD34+ cells were always present as shown by cytochemistry and immunocytochemistry, as well as fetal cells as shown by immunocytochemistry and FISH.

Monoclonal antibodies to ε- and γ-chain-Hb detect only a portion of fetal cells present on the slide, we therefore also used a monoclonal antibody to i-antigen [42], which is expressed also by non-erythroid cells and has already been

Table 6
Investigation of pathological samples

Gestational age	Invasive procedure	Karyotype	No. of cells aneuploid by FISH
Samples after invasive procedure			
MGT 17 weeks	amniocentesis	Trisomy 21	75
LZN 18 weeks	amniocentesis	Trisomy 21	110
BDN 17 weeks	amniocentesis	Trisomy 21	129
DMN 17 weeks	amniocentesis	Trisomy 21	82
MNC 12 weeks	CVS	Trisomy 21	132
DAG 20 weeks	amniocentesis	Trisomy 13	98
COL 15 weeks	CVS	Trisomy 21	425
BRA 17 weeks	amniocentesis	Trisomy 18	48
Samples before invasive procedure			
CLB 10 weeks	CVS	Trisomy 21	40
ZNT 10 weeks	CVS	Trisomy 21	83
ABT 9 weeks	CVS	Triploidy	24
RSN 14 weeks	amniocentesis	Trisomy 21	62
LVG 11 weeks	CVS	Triploidy	97
MNS 14 weeks	amniocentesis	Trisomy 13	24
MLN 13 weeks	amniocentesis	Trisomy 21	84
MOD 14 weeks	amniocentesis	Trisomy 21	48

50,000 nuclei have been scored for FISH signals. Controls examined in parallel by FISH showed a percentage of false-positive trisomic cells in 0.012% when 50,000 nuclei were scored.

used in the old literature for successful prenatal genetic diagnosis [43]. Cells positive for i-antigen were always present in our isolated cell fractions.

Immunocytochemical investigations in the present study shows that ε-chain-Hb-positive cells are still present in 2nd trimester blood samples (Table 3). The switch from ε- to γ-chain-Hb is completed between the 14th and 18th weeks of gestation, which is later than previously believed [44].

The number of NRBCs for genetic analysis is much higher when detection is based on both ε- and γ-chain-Hb, although ε-chain-Hb is considered a more reliable marker of fetal cells than γ-chain-Hb [45]. In conclusion, the MoAb for ε-chain-Hb has a high specificity but detects only a part of fetal NRBCs, while the use of MoAb for γ-chain-Hb detects not only fetal NRBCs, but also some maternal NRBCs. Both of them do not detect fetal stem cells. The use of MoAb for i-antigen detect fetal cells belonging to both cell type, fetal NRBCs and fetal stem cells, but also a very small population of apparently maternal lymphocytes.

An unresolved difficulty with this approach is the inability to distinguish a fetal cell unequivocally as a fetal cell before the analysis of its chromosome constitution. Combining FISH evaluation with fetal cell in situ identification through a fluorescent cell marker would solve both these inconveniences [46]. This approach is presently in progress in our laboratory using monoclonal antibody for i-antigen.

In conclusion, many different procedures have been suggested to isolate fetal cells from maternal blood, requiring highly sophisticated equipment and/or technically demanding technologies. We here provide evidence that fetal cells can always be obtained from maternal blood in sufficient number for prenatal non-invasive genetic investigation, using simple laboratory equipment and procedure.

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